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CRISPR/Cas12a-RPA integrated assay for potential rapid detection of *Fusarium* spp.

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Abstract. Timely detection and accurate identification of crop pathogens are considered fundamental components of sustainable agricultural systems alarming prompt and effective crop protection management. The cutting-edge CRISPR/Cas12a technology is emerging as a nucleic-acid-based platform with high potential for rapid, sensitive, and specific phytopathogen detection. In this study, we aimed to develop a CRISPR/Cas12a-based method to detect *Fusarium* spp., targeting the acl1 gene. A plasmid carrying the target gene fragment was successfully amplified using Recombinase Polymerase Amplification (RPA), confirming the feasibility of integrating this isothermal amplification with the CRISPR/Cas12a detection method. Optimization of the CRISPR-Cas12a reaction revealed optimal conditions at 100 nM MbCas12a, 100 nM crRNA, and 5 μM ssDNA reporter. Sensitivity assays demonstrated reliable detection of the *acl1* gene up to 1:1000 dilution. Specificity evaluation involving different plant-pathogenic fungal species confirmed the high specificity of the developed method. Altogether, this study highlights the potential of the CRISPR/Cas12a-based system as a promising diagnostic approach for agricultural applications to detect *Fusarium* spp.
Keywords: CRISPR, Cas12a, *Fusarium,* Recombinase Polymerase Amplification, nucleic acid detection

Introduction

Food Security is one of the 17 Sustainable Development Goals (SDGs) that require substantial efforts to address it. Sustainable agricultural production is challenged by different factors, including plant diseases caused by diverse pathogens [1,2]. The global economic loss attributed to plant diseases is estimated at approximately 40 billion USD annually, emphasizing the urgent need for efficient and sustainable crop protection strategies [2]. According to the Bureau of National Statistics of Kazakhstan (https://stat.gov.kz/), cereals, particularly wheat, occupy the largest share of cultivated land, accounting for approximately 68-72% of total sown areas in

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2022–2023. However, combating factors that limit crop productivity remains a critical challenge in agriculture, with biotic stresses such as pathogenic diseases posing significant threats to yield.

Fusarium spp. are considered one of the most destructive plant pathogens, affecting a wide range of agricultural crops worldwide [3]. Species within this genus infect wheat, barley, rice, tomato, and potato, causing severe yield reductions and producing toxic secondary metabolites known as mycotoxins. Diseases like *Fusarium* head blight and root rot cause billions of dollars in crop losses per annum. For instance, wheat rust and Fusarium infections alone are responsible for 5 and 3 billion USD in annual losses, respectively [4].

Rapid and precise detection of plant pathogens is essential for sustainable agriculture and effective crop protection [5]. Conventional detection methods include morphology- or PCR-based approaches [6]. Such methods are often time-consuming and require well-equipped laboratories, limiting their applications for rapid in situ diagnostics. Consequently, precise, nucleic-acid-based, portable detection methods attract considerable interest as alternative methods for validating plant infections under field conditions [5,6].

In recent years, molecular biology tools have advanced significantly [7], and CRISPR/Cas systems have emerged as powerful tools not only for genome editing but also for sensitive and specific pathogen detection [8,9]. Among CRISPR-associated nucleases, Cas12a represents remarkable potential for diagnostics due to its ability to recognize specific DNA sequences, process its own guide RNA (crRNA), and exhibit trans-cleavage activity toward single-stranded DNA once activated by the target sequence [10–12]. Such characteristics are crucial for developing portable, rapid, and sensitive detection platforms for agricultural pathogens. Moreover, isothermal amplification techniques, like Recombinase Polymerase amplification (RPA) [13], were developed to enable amplification of target genetic loci at relatively low temperatures without the need for advanced laboratory equipment. Such isothermal amplification methods integrated with CRISPR/Cas12a remarkably expand their application potential as highly sensitive and field-deployable assays [14]. Additionally, potential coupling with lateral flow devices or portable fluorescence readers for real-time visualization underscores their potential for monitoring disease outbreaks in remote agricultural fields as well as for implementing early intervention strategies and applying appropriate management strategies [15,16].

Recent studies already demonstrated the potential of CRISPR/Cas12a for detecting various agricultural pathogens [9]. This approach was successfully applied for the detection of *Alternaria solani* [17], *Puccinia striiformis* f. sp. tritici [18], and *Phytophthora ramorum* [19]. *Regarding Fusarium,* the CRISPR/Cas12a method was reported for identifying *Fusarium circinatum* [20], *Fusarium asiaticum* [21], and *Fusarium graminearum* [22] in different crops. Nevertheless, further research is required to extend this approach to other agriculturally significant Fusarium species. Therefore, the objective of the present study was to develop a CRISPR/Cas12a-based method for the detection of Fusarium spp.

Materials and research methods

Selection of genetic locus, design of RPA primers, and crRNA

The sequence of the *acl1* gene from the *Fusarium tricinctum* strain KAS:1036 (accession number JX397819.1) was selected as the target locus. RPA primers were designed with a melting temperature of 40–60 °C, GC content of 40–60%, and length of 30–35 bp. crRNA was designed based on a TTTG PAM site. All designs were performed using VectorNTI software. crRNA was transcribed *in vitro* using the HiScribe T7 Quick High Yield RNA Synthesis Kit and purified with the Monarch RNA Cleanup Kit (New England Biolabs) according to the manufacturer's instructions.

Identification of fungal isolates

Preliminary morphology-based identification, microscopy observation, DNA extraction, and ITS-based identification were as reported in our previous study [23].

Construction of the positive control carrying the acl1 gene and RPA validation

Positive control plasmids were obtained using the NEB PCR cloning kit according to the manufacturer's instructions. Successfully cloned plasmids were purified with the GeneJET Plasmid Miniprep Kit. RPA was performed following TwistAmp Basic kit official protocols (TwistDX, Cambridge, United Kingdom). Negative control was H2O throughout all relevant studies.

Optimization of CRISPR/Cas-12a-based reaction

Various concentrations of MbCas12a (10 nM, 50 nM, 100 nM, 250 nM, and 500 nM), crRNA (10 nM, 50 nM, and 100 nM), and ssDNA reporter (1.0, 2.0, 3.0, 4.0, and 5.0 μM) were tested to determine the optimal conditions. Recombinant MbCas12a protein obtained from *Moraxella bovis* in our laboratory, as described previously [24], was verified by reverse-phase C18 liquid chromatography-tandem mass spectrometry (LG-MS/MS) and employed in all corresponding studies.

Cis-activity of MbCas12a toward the acl1 gene

Cis-activity of MbCas12a was assessed using purified PCR amplicons (QIAquick PCR Purification kit, QIAQEN GmbH, Hilden, Germany) of the acl1 target sequence, amplified with RPA-designed primers (described above). MbCas12a was incubated with crRNA to form a ribonucleoprotein complex, followed by the addition of the PCR product and further incubation at 37°C. Cleavage of the target amplicon was evaluated via horizontal agarose gel electrophoresis and visualized using a GelDoc system.

Sensitivity validation and specificity verification

Positive control plasmid was serially diluted (1:1, 1:10, 1:100, and 1:1000) and amplified via RPA. Amplicons were analyzed by gel electrophoresis and fluorescence assays.

Various fungal plant pathogens commonly hosted wheat, such as *Nigrospora oryzae, Bipolaris sorokiniana,* and 2 strains of *Alternaria spp.*, were included to evaluate specificity. All samples were amplified with RPA and analyzed using agarose gel and naked-eye fluorescence readout.

Results

Selection of genetic locus, design of RPA primers, and crRNA

Based on the sequence of the acl1 gene available in the NCBI database (accession number JX397819.1), one set of RPA primers was designed according to the parameters described above. The forward (RPA-Phyto-Fus-acl1) and reverse primers (RPA-Phyto-Fus-acl1-RV) were GTGTCTTGAGACAATTCCTCGTTGAGCCTT and TAATGAAGTCGACAAGGACGTTGTGGACAC, respectively. The crRNA (Figure 1) was designed according to the TTTG PAM site in the *acl1* sequence (MbCas12a-crRNA-Fus-*acl1*: ggactctcgtctactggcgaggaaATCTACAAACAGTAGAAATTCC CTATAGTGAGTCGTATTAGAATT).

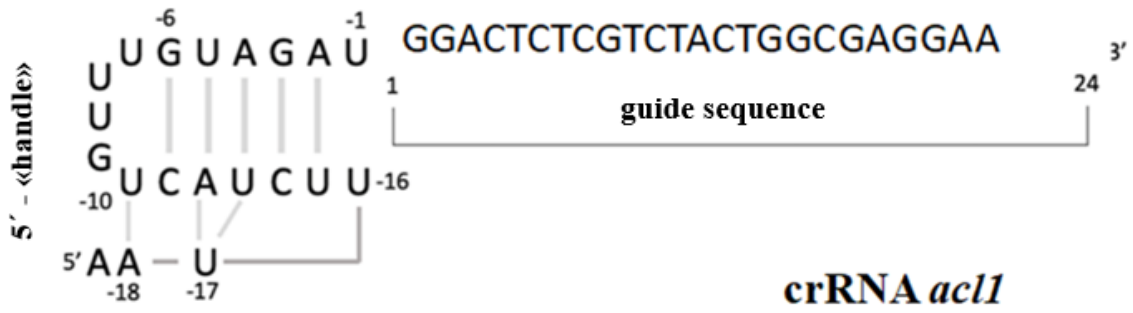

Figure 1. Design of the guide RNA targeting the acl1 gene

Identification of fungal isolates

A total of 12 fungal strains were isolated from cereal seeds samples [23]. BLAST analysis of ITS sequences revealed that eight strains (4/1, 7/2, 8/1, 8/5, 8/7, 11/1, 41/1, and 42/1) belong to *Alternaria* spp. and their sequences were deposited in NCBI database under accession numbers PQ056909-PQ056916, respectively. The remaining four strains – 22/1, 465, 1/3, and 25/1 - deposited as PQ056917-PQ056920 were identified as *Nigrospora oryzae, Bipolaris sorokiniana, Fusarium equiseti,* and *Fusarium acuminatum,* respectively. The *Fusarium* strain 25/1 was selected to validate the specificity of the designed RPA primers.

Construction of a positive control carrying the acl1 gene and RPA validation

Amplification of the Fusarium acuminatum 25/1 strain confirmed the functionality of the designed RPA primers (Figure 2a). The obtained PCR product was cloned into the pJET cloning kit (Thermo Scientific), yielding four positive clones out of five tested (Figure 2b)

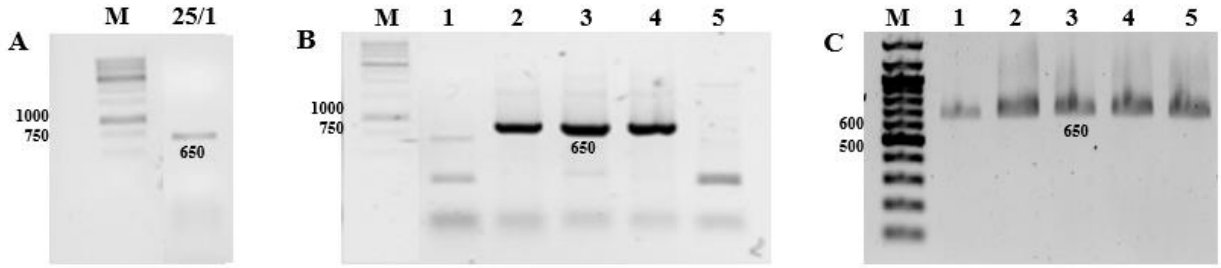

Figure 2. Construction of the plasmid carrying the *acl1* gene: (**a)** PCR amplification of the *acl1* gene from Fusarium strain 25/1 using RPA primers; (**b**) number of E. coli clones carrying plasmids with the cloned *acl1* gene; (**c**) RPA-based amplification of the *acl1* gene in the positive control (five technical copies)

The plasmid containing the cloned acl1 fragment was purified and subsequently used as a positive control in all subsequent assays.

Since isothermal amplification is the crucial element to perform the CRISPR/Cas12a methods without thermal equipment, positive control was amplified using RPA to validate this amplification method for obtaining amplicons required by the subsequent assays. RPA

amplification of the positive control in five technical replicates produced consistent amplicon yields (Figure 2c), validating the suitability of RPA for generating sufficient template DNA for CRISPR/Cas12a detection.

Optimization of CRISPR/Cas-12a-based reaction

The crucial elements of the CRISPR/Cas12a reaction include MbCas12a, crRNA, and ssDNA reporter. Various concentrations of MbCas12a (10-500 nM), crRNA (10 nM-100 nM), and ssDNA reporter (1.0-5.0 μM) were evaluated based on end-point fluorescence after 30 minutes of incubation (Figure 3).

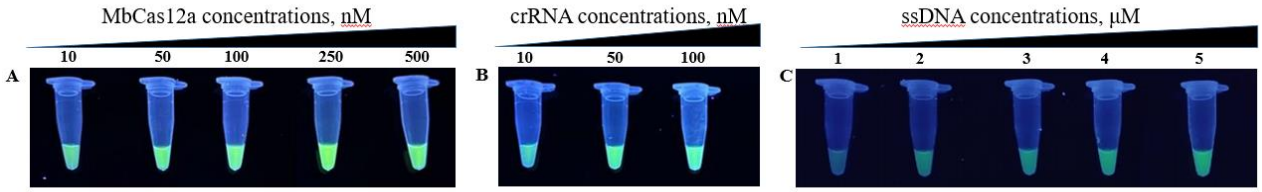

Figure 3. Optimization of CRISPR/Cas12-based reaction: (**a**) effect of MbCas12a concentration; (**b**) effect of crRNA concentration; **(c)** effect of ssDNA reporter concentration

Optimal signal intensity for the naked eye was achieved using 100 nM MbCas12a, 100 nM crRNA, and 5.0 μM ssDNA reporter.

Cis-activity of MbCas12a toward the acl1 gene

The cis-activity of MbCas12a, i.e., its ability to cleave the target site guided by the designed crRNA, was studied using the purified *acl1* PCR product (~650 bp). Partial cleavage was observed across all reaction durations ranging from 5 to 60 min (Figure 4).

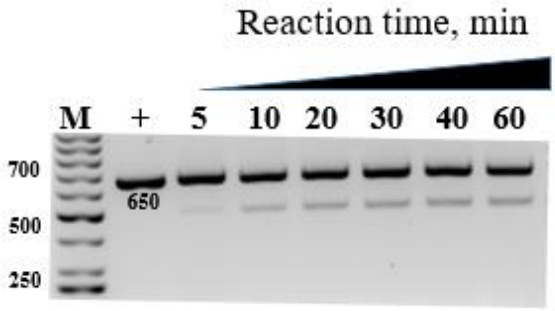

Figure 4. Evaluation of MbCas12a cis-activity using 650 bp acl1 PCR amplicon. The + lane represents the uncleaved control (PCR amplicon without MbCas12a reaction), whereas lanes 5–60 min correspond to reaction time intervals

Sensitivity validation and specificity verification

The analytical sensitivity and specificity of the developed method were evaluated using serial dilutions of the plasmid template and different fungal DNA samples, respectively. Both conventional gel electrophoresis and fluorescence signal with the naked eye assays demonstrated a sensitivity level up to a 1:1000 dilution of the origin plasmid (Figure 5a, b).

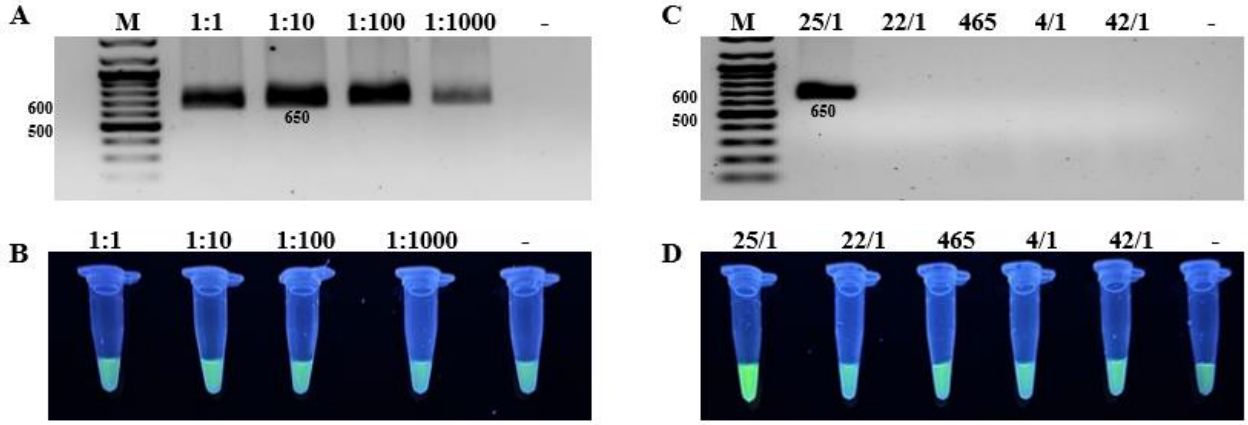

Figure 5. Evaluation of assay sensitivity and specificity. **(a**) sensitivity: gel electrophoresis results; serial dilutions of the target DNA fragment (1:10 to 1:1000); the minus sign indicates the negative control (H2O). **(b**) sensitivity: fluorescence assay results (legend as in panel a). **(c)** specificity: lanes 25/1, 22/1, 465, 4/1, and 42/1 correspond to Fusarium spp. positive controls: N. oryzae, B. sorokiniana, and Alternaria spp. were tested for cross-reactivity; the minus sign indicates the negative control (H2O). (**d**) Specificity: fluorescence assay results (legend as in panel c)

Specificity examination confirmed high selectivity of the assay toward the acl1 gene: none of the non-Fusarium fungal isolates was amplified during RPA reaction (Figure 5c). In the fluorescence assay, only the positive control exhibited visible fluorescence, further validating assay specificity (Figure 5d).

Discussion

The CRISPR/Cas system, originally discovered in bacteria and archaea as a form of adaptive immunity, has rapidly developed into a powerful platform for molecular diagnostics [25]. Since the first observation of clustered repeats in E. coli by Ishino et al. in 1987 [26], successive studies have revealed the system's biological function and its application potential, including diagnostics of various pathogenic agents [8,9,25].

Among the Cas nucleases, Cas12a is particularly attractive for diagnostic purposes due to its RNA-guided DNase activity and unique collateral (trans) cleavage property, which allows for the detection of target DNA sequences through a simple fluorescence readout [10,27,28]. Cas12a recognizes specific DNA targets guided by crRNA, and upon activation, it non-specifically cleaves single-stranded DNA molecules such as fluorescent reporters [29,30]. This mechanism forms the basis for highly sensitive detection systems that can operate under isothermal conditions. When combined with recombinase polymerase amplification (RPA), which efficiently amplifies DNA at constant temperatures, the Cas12a system can detect even minimal amounts of pathogen DNA without requiring thermal cyclers or complex laboratory equipment [15]. Such features make it a promising platform for rapid and field-adapted diagnostics.

Several recent studies have demonstrated the potential of Cas12a-based diagnostics for plant pathogens. For example, RPA–CRISPR/Cas12a systems have been developed for Diaporthe aspalathi and D. caulivora causing soybean stem canker [31], Verticillium dahliae associated with wilt disease [32], and Leptosphaeria maculans responsible for phoma stem canker in oilseed rape [30]. These methods achieved detection within 30–60 minutes and with

sensitivities as low as a few DNA copies, showing clear applicability in agricultural pathogen monitoring. Furthermore, CRISPR/Cas12a has been successfully used to identify several plant viruses, including tomato mosaic virus, tomato brown rugose fruit virus, and beet necrotic yellow vein virus, demonstrating its versatility across taxonomic groups [33,34].

In the present study, we applied this concept to develop a CRISPR/Cas12a-based diagnostic assay targeting the acl1 gene to detect *Fusarium*spp. To the best of our knowledge, this is the first report describing a CRISPR/Cas12a-based detection system for *Fusarium* spp. developed in Kazakhstan. The designed RPA primers and crRNA enabled specific recognition of the target fragment, with optimal reaction parameters determined for Cas12a, crRNA, and ssDNA reporter concentrations. The assay showed both high specificity and remarkable sensitivity, detecting target DNA dilutions up to 1:1000 of the original plasmid. Importantly, the system distinguished Fusarium species from other common cereal-associated fungi such as *Alternaria spp., Bipolaris sorokiniana,* and *Nigrospora oryzae.*

Altogether, our results support the potential of Cas12a-based systems for accurate, portable, and cost-effective detection of fungal pathogens. By enabling on-site diagnostics, such approaches could facilitate early detection and management of *Fusarium*-related diseases, helping to reduce crop losses and improve yield stability. Continued optimization of reaction conditions and integration into simple field-deployable formats, such as lateral flow strips or smartphone-based fluorescence readers, will further expand the practical use of this method in agricultural monitoring and plant health management.

Conclusion

The CRISPR/Cas12a-based method targeting the *acl1* genetic locus was developed to detect Fusarium spp. with high specificity and sensitivity. Integrated with the isothermal Recombinase Polymerase Amplification and a fluorescence assay, this method demonstrates strong potential for rapid and reliable detection of *Fusarium* pathogens under field conditions. The proposed system can contribute to improved plant disease diagnostics and sustainable crop protection practices.

Author Contributions

All authors contributed to the article and approved the submitted version. **A.Sa., Zh.Zh., A.Z. –** formal analysis, investigation, writing – original draft preparation; **A.Sh., M.A., S.A. –** methodology, conceptualization, discussion, writing – review and editing; **S.A., A.Z.** – funding acquisition, project administration, data curation, supervision.

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Conflicts of Interest

The authors declare the absence of any conflict of interest.

Compliance with ethical standards

This article does not contain a description of studies performed by the authors involving people or using animals as objects.

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Fusarium spp. жылдам анықтауға арналған CRISPR/Cas12a-RPA біріктірілген талдау әдісінің потенциалы

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Аңдатпа. Егін ауруларын дер кезінде анықтау мен дәл идентификациялау – ауыл шаруашылығындағы тұрақты жүйелердің негізгі құрамдас бөлігі болып табылады және өсімдіктерді жедел әрі тиімді қорғауды қамтамасыз етеді. Алдыңғы қатарда саналатын CRISPR/Cas12a жүйесі фитопатогендерді жоғары дәлдікпен, сезімталдықпен және жедел анықтауға мүмкіндік беретін нуклеин қышқылдарына негізделген заманауи платформа ретінде қарқынды дамып келеді. Осы зерттеуде біз Fusarium туысының саңырауқұлақтарын анықтауға арналған acl1 генін нысана еткен CRISPR/Cas12a негізіндегі әдісті әзірледік. Нысана ген фрагментін қамтитын плазмида Рекомбиназалық полимеразалық амплификация (RPA) әдісімен сәтті амплификацияланды, бұл изотермиялық амплификацияны CRISPR/Cas12a детекция әдісімен біріктірудің орындылығын растады. CRISPR-Cas12a реакциясын оңтайландыру нәтижесінде ең тиімді шарттар 100 нМ MbCas12a, 100 нМ crRNA, және 5 μM ssDNA репортері ретінде анықталды. Сезімталдықты анықтау сынақтары acl1 генін 1:1000 дейінгі сұйылтуда сенімді анықтауға болатынын көрсетті. Түрлі өсімдік патогенді саңырауқұлақтар түрлерін қамтыған ерекшелік сынақтары әзірленген әдістің жоғары ерекшелігін дәлелдеді. Осылайша, бұл зерттеу Fusarium туысының өкілдерін анықтау үшін CRISPR/Cas12a негізіндегі жүйенің ауыл шаруашылығында қолдануға арналған перспективалы диагностикалық құрал екенін көрсетеді.
Түйін сөздер: CRISPR, Cas12a, *Fusarium,* Рекомбиназалық полимеразалық амплификация, нуклеин қышқылдарын анықтау

CRISPR/Cas12a-RPA интегрированный метод для потенциала быстрой детекции *Fusarium* spp.

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Аннотация. Своевременное обнаружение и точная идентификация возбудителей болезней сельскохозяйственных культур являются ключевыми элементами устойчивых агросистем, обеспечивающими оперативное и эффективное управление защитой растений. Современная технология CRISPR/Cas12a активно развивается как платформа на основе нуклеиновых кислот с высоким потенциалом для быстрого, чувствительного и специфичного выявления фитопатогенов. В данной работе разработан метод на основе CRISPR/Cas12a для детекции грибов рода Fusarium, нацеленный на ген acl1. Плазмида, содержащая фрагмент целевого гена, была успешно амплифицирована с использованием метода Рекомбиназной полимеразной амплификации (RPA), что подтвердило возможность интеграции этой изотермической

амплификации с методом детекции CRISPR/Cas12a. Оптимизация реакции CRISPR-Cas12a показала, что наилучшие условия достигаются при концентрациях 100 нМ MbCas12a, 100 нМ crRNA и 5 μM ssDNA-репортера. Тесты чувствительности показали надёжное выявление гена acl1 при разведении до 1:1000. Оценка специфичности с участием различных фитопатогенных грибов подтвердила высокую селективность разработанного метода. В целом результаты исследования демонстрируют потенциал системы на основе CRISPR/Cas12a как перспективного диагностического инструмента для выявления грибов рода Fusarium в сельскохозяйственных применениях.

Ключевые слова: CRISPR, Cas12a, *Fusarium,* Рекомбиназная полимеразная амплификация, детек-ция нуклеиновых кислот

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